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THE DISTRIBUTION OF PHOSPHOARGININE AND PHOSPHOCREATINE IN MARINE INVERTEBRATES[1]

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Despite the wide morphological, ecological and functional diversity of the two or more million species of animals which exist on earth, comparative biochemical studies emphasize the basic similarity, at the molecular level, of all animal life. This is true both as regards chemical replicating mechanisms, on the one hand, and intracellular enzyme and co-enzyme systems which are particularly concerned with energy transfer, storage, and utilization, on the other.

The phylogenetic significance of the high energy phosphagens, phosphocreatine and phosphoarginine, was first suggested by the studies of Kutscher and Ackermann (1926) who proposed the principle that creatine was the phosphagen of vertebrate muscle and that invertebrates should therefore be termed "acreatinate." More recent studies by Yudkin (1954) and by Roche, Thoai and Robin (1957), who identified creatine phosphate as present in three other invertebrate phyla, have cast doubt on the original concept of Kutscher and Ackermann, and the inferences of Needham, Baldwin and Yudkin (1932) and Baldwin and Needham (1937), as to the phylogenetic implications of differential distribution of the two phosphagens. A recent review of Ennor and Morrison (1958) covered the scattered, later reports on the distribution of phosphocreatine in marine organisms. They concluded (page 665) that "phosphocreatine and phosphoarginine cannot be regarded as characteristic of the vertebrates and invertebrates, respectively, for, while phosphocreatine *is* a phosphagen characteristic of the vertebrates, no one phosphagen is characteristic of the invertebrates."

However, even the early work of Needham (1932) *et al.*, showed that creatine did occur in some of the echinoderms as well as the hemichordates, with the inference on their part, that this was evidence for a common ancestry for the echinoderms and the vertebrates. Other biochemical evidence by Bergmann, McLean and Lester (1943) and Bergmann (1949) and more limited embryological evidence by Breneman (1966) have also linked the echinoderms phylogenetically to the primitive chordates, particularly the hemichordates. However, the techniques of separation and identification of these two phosphagens, which have been employed in these earlier studies and upon which phylogenetic inferences were based, are either questionable or differ from one another significantly.

The present study was therefore undertaken in an attempt to resolve the question of the possible phylogenetic interrelationship of the echinoderms and primitive chordates, suggested both by embryological and by Bergmann's quite different biochemical line of evidence. Accordingly, the distribution of the two phosphagens in representative species of four major classes of the Echinodermata

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and in one common hemichordate was examined by more sophisticated methods of extraction and separation, and their identification by much more sensitive, analytical procedures than had been employed by most earlier workers.

Materials and Methods

Determinations were made on the longitudinal muscles of the North Atlantic common sea cucumber, *Thyone briareus,* and the larger South Atlantic sea cucumber, *Ludwigthuria floridana,* on the dorsal skin of the North Atlantic starfish, *Asteria forbesi,* and of the local Florida starfish, *Echinaster sentus,* the complete rays of the brittle star, *Ophioderma brevispina,* the lantern retractors of the sea urchin, *Arbacia punctata,* the unfertilized eggs of *A. punctata* and of the Southern sea urchin, *Lytechinus variegatus,* the unfertilized eggs of *A. forbesi,* and finally, total body homogenates of the hemichordate, *Saccoglossus kowalevskii.*

Tissues were excised fresh, rinsed in sea water, quickly dried on toweling, weighed, and frozen in liquid nitrogen-cooled Elvehjem-Potter mortar with a stainless steel pestle. The homogenate was decanted and the homogenizer cup rinsed in cold 0.4 N perchloric acid into a cold stainless steel centrifuge tube. This was then centrifuged at 5° C, at 5000 rpm (3200 *g*) for ten minutes. The supernatant liquid so obtained was neutralized with cold 5 *N* NaOH and then quantitatively diluted to a known volume (10 ml) with cold, doubly-distilled water.

Arbacia eggs were collected by low voltage electrical stimulation and starfish eggs by means of the shedding hormone of Chaet (1966), in each case into 4 ml of cold doubly distilled water. After ten minutes to permit complete cytolysis, 4 ml of cold 0.8 *N* perchloric acid was added to each 4 ml of egg suspension. This mixture was then neutralized with cold 5 *N* NaOH and centrifuged cold at 10,000 rpm (12,800 *g*) and the supernatant liquid decanted and made to a known final volume with cold, doubly-distilled water.

Inorganic phosphate was determined by the method of Lowry and Lopez (1946) for "before" and "after" acid hydrolysis samples.

Appropriate acid hydrolysis consisted of heating known aliquots of neutralized brei, re-acidified with 0.2 *N* NaCl (1:1). For phosphocreatine hydrolysis, this involved nine minutes' exposure at 65° C. For phosphoarginine hydrolysis, the acidified brei was exposed for one minute in a boiling water bath. In each case, the previously heated acidified brei was cooled rapidly, prior to their separation and identification.

The techniques used in isolating and identifying the two guanidines include differential elution column chromatographic ion-exchange method for their separation and their identification and estimation by critical modifications of the Sakaguchi (arginine) and diacetyl (creatine) reactions. In each experiment, furthermore, a check on the procedure was made by side-by-side isolation and identification of creatine and arginine from standard solutions run through columns and similarly identified in parallel with the experimental biological material.

Known volumes of the cooled "before" hydrolysis samples were passed through one set of (three) 2″ × ⅝″ Amberlite® columns and "after" hydrolysis samples through a second series of (three) similar columns, according to the method of Anderson, Williams, Krise and Dowben (1957), as follows:

Samples are poured into the first column containing previously-washed, strong anion exchange resin, IRA-401, and eluted at a rate of 1 ml per minute. This column retains most impurities but elutes both arginine and creatine upon washing with doubly-distilled water.

Column 2 contains IRC-50, a weak cation exchange resin, which binds the arginine but permits creatine to be freed upon elution with doubly-distilled H_2O.

Column 3 contains IR-120, a strong cation exchange resin, which holds the creatine.

Arginine is then eluted from the second (IRC-50) column with 50 ml of 2 *N* sodium acetate (pH 11.5) and the creatine similarly eluted with an identical volume of the same sodium acetate solution from the third (IR-120) column. In each case the eluted guanidine is collected into a 50 ml flask, at a flow rate of one ml per minute.

Arginine analysis

Arginine is determined quantitatively by the Sakaguchi method, as modified by Rosenberg, Ennor and Morrison (1956). A mixture of 2 ml of sulfosalicylic acid-oxime, 2 ml of the eluted sample and 1 ml of 2.5% NaOH is set aside in ice for 15 minutes and then warmed for 45 sec in room temperature tap water. One ml of hypobromite solution is then added and the optical density of the resulting solution is read immediately at 500 mμ in the B&L Spectronic 20.

Creatine analysis

This is the alkaline diacetyl-naphthol method of Barritt as adapted by Anderson *et al.* (1957), and Dubnoff (1957). The test mixture includes 1 ml of stock alkali, 1 ml of alpha-naphthol solution, 4 ml of the sample—previously adjusted to a pH of 9–10, with 5 *N* NaOH (approximately 0.4 ml of 5 *N* NaOH/25 ml sample) (or 4 ml of the standard or of doubly-distilled water) plus 1 ml of (Sigma) para-hydroxymercuribenzoate (3.6 g/200 ml). To this mixture is added 1 ml of diacetyl solution (1 ml of 1% stock solution which has been diluted 1–20 times in doubly-distilled water) and the optical density is read exactly 20 minutes afterwards at 525 mμ in the B&L Spectronic 20.

In each case replicates were determined for three "before" hydrolysis and three "after" hydrolysis samples and for three arginine and three creatine standards (0.25 mg/ml, diluted 1:50), all read against corresponding, doubly-distilled water blanks.

Results

All data shown in Table I represent determinations made from specimens obtained fresh from the sea, *i.e.*, not stored over any period of time prior to isolation and determination of the phosphagens involved. Figures in parentheses indicate the number of positive tests for the guanidine or its phosphagen, out of the total number of experiments performed.

Results obtained clearly show that moderate levels of creatine phosphate occur in nine species of echinoderms, representing four of the five major classes of that

TABLE I

Distribution of arginine and creatine and their phosphagens in marine invertebrates

Specimens	Arginine	Arginine-PO_4	Creatine	Creatine-PO_4
Thyone briareus longitudinal muscles	± (8/11)	++++ (9/11)	± (8/11)	++++ (11/11)
Ludwigthuria floridana longitudinal muscles	++ (34/45)	++ (33/45)	+ (22/45)	+++ (31/45)
Asterias forbesi rays	± (10/18)	+ (13/18)	± (9/18)	++ (13/18)
ova	− (0/7)	− (0/7)	± (1/7)	− (0/7)
Asterias vulgaris rays	± (6/6)	+ (3/6)	± (1/6)	++ (5/6)
Echinaster sentus rays	+ (20/49)	+ (18/49)	+ (23/49)	++ (24/49)
Echinaster spinulosa rays	+ (2/5)	+ (3/5)	+ (3/5)	+++ (4/5)
Ophioderma brevispina rays	++ (1/6)	+ (1/6)	+++ (6/6)	+++ (5/6)
Arbacia punctata lantern protractors	− (0/13)	+ (1/13)	+++ (8/13)	+++ (12/13)
ova	− (0/8)	± (3/8)	− (0/8)	± (5/8)
Lytechinus variegatus ova	+ (5/7)	++ (2/7)	+ (3/7)	++ (5/7)
Saccoglossus kowalevskii	± (5/7)	++ (3/7)	± (2/7)	+ (1/7)

N.B. ± = 0 − 0.09 μeq/gram
+ = 0.10 − 0.49 μeq/gram
++ = 0.50 − 0.99 μeq/gram
+++ = 1.0 − 1.9 μeq/gram
++++ = 2.0 − μeq/gram

phylum. What is more significant is that these data were obtained from muscles in three studies and from tissues which are heavily muscularized, but from which excision of muscles *per se* is a technically difficult task, in the case of the remaining six species studied. Free creatine, on the other hand, was demonstrable in only small to trace quantities in all but two species, *viz.*, the rays of the brittle star, *Ophioderma*, and the purple sea urchin, *Arbacia*, lantern protractors showing relatively high amounts of that free guanidine.

The so-called "phosphagen of the invertebrates," arginine phosphate, occurred in moderate to high amounts only in the muscles of the two sea cucumbers studied,

Thyone and *Ludwigthuria*. Moreover, this phosphagen and its free guanidine, was virtually absent from the lantern protractors or muscles of the echinoid, *Arbacia*, while being present in the unfertilized ova of both this sea urchin and the Southern form, *Lytechinus*. As for the hemichordate, *Saccoglossus*, only light to moderate amounts of *both* phosphagens were found in fewer than half of the experiments performed.

Discussion

These data are somewhat at variance from a number of earlier reports, summarized in the review by Ennor and Morrison (1958).

In this connection, further experiments might well be undertaken to confirm the identity of the phosphagen involved by the presence or absence of the appropriate (creatine or arginine) phosphokinase. However, as Ennor and Morrison (1958) point out, such experiments would assume a substrate specificity for each phosphokinase and for each species studied. Furthermore, such a series of experiments would require rather extended enzymatic studies of the optimal conditions for estimating enzyme activity for each species involved, as well as their substrate specificity characteristics. Collection of such data at this time would inordinately delay the publication of our own data, obtained by reliable and competent methods over a period of several years.

For the asteroids, we found, unlike Needham *et al.* (1932) (who reported only phosphoarginine in the starfish, *Marthasterias glacialis*, *moderate* amounts of both phosphocreatine and phosphoarginine) in the closely related species, *Asterias vulgaris*. Arnold and Luck (1933) also reported only *arginine* in several species of starfish, but by a method which suggested that this guanidine must be occurring as the phosphagen, rather than by direct determination of phosphocreatine.

As for the holothuroids, Kutscher and Ackermann (1926) reported only arginine in *Holothuria* and *Leptosynapta* and Meyerhoff (1928) only phosphoarginine in *Stichopus*. Baldwin and Needham (1937) inferred the presence of phosphoarginine in *Holothuria* sp., from their ability to synthesize the phosphagen from a solution of arginine, inorganic phosphate and muscle extract. Conversely, they assumed the *absence* of phosphocreatine from the *inability* of such extracts to synthesize the phosphagen from creatine and inorganic phosphate. Likewise, by indirect methods quite different from ours, both Baldwin and Yudkin (1950) and, more recently, Stephens, Van Pilsum and Taylor (1965) reported the absence of phosphocreatine in (what appears to be total body homogenates of) *Thyone briareus*. On the other hand, Verzhbinskaya, Borsuk and Kreps (1935), did report the presence of both phosphagens in the holothuroid, *Cucumaria frondosa*.

The virtual absence of both arginine and phosphoarginine in both echinoids studied, with rather appreciable amounts of creatine and phosphocreatine in *Arbacia punctata*, are in strong contrast to data of earlier reports. Thus, both phosphocreatine and phosphoarginine were reported by Arnold and Luck (1933) in *Strongylocentrotus franciscanus*, by Needham *et al.* (1932) in *Strongylocentrotus lividus* jaw muscles and by Baldwin and Yudkin (1950) in *Echinus esculentus* jaw muscles. However, all of the data by Baldwin and Yudkin (1950) were based on methods described by the authors themselves (page 617), as follows:

"although neither of these reactions is very specific, useful indications were nevertheless obtained"(?). Griffiths, Morrison and Ennor (1957) also reported both phosphagens in one echinoid, *Heliocidaris erythrogramma,* but only phosphoarginine in *Centrostephanus rodgersii,* which further indicates the relative variability of distribution of the two phosphagens, even within a single class of echinoderms.

In the brittle star, *Ophioderma brevispina,* only light to moderate amounts of arginine and arginine phosphate were found in only one of the six specimens studied, which compared favorably with the report of Baldwin and Yudkin (1950), who could detect only creatine phosphate in the arms and discs of *O. brevipina* and *O. longicauda.*

But for the primitive chordate, our own seven studies of *Saccoglossus kowalevskii* showed only traces of arginine, the presence of arginine phosphate in moderate amounts in at least three cases, and only traces to very light amounts of creatine and creatine phosphate in not more than two cases studied. This again is in contrast to the data of Baldwin and Yudkin (1950) in which only creatine phosphate could be reported by their methods, for the same species. Needham *et al.* (1932), on the other hand, reported both creatine and arginine phosphate, in the related hemichordate, *Balanoglossus salmoneus,* only creatine phosphate in the solitary urochordate, *Styela carnea,* but *neither phosphagen* in two other urochordates, the solitary form, *Ciona intestinalis,* and the colonial *Amaroucium constellatum.* Morrison, Griffiths and Ennor (1956) similarly reported the unequivocal absence of both phosphoarginine and the enzyme arginine phosphokinase, as well as arginine itself; on the other hand, they conclusively demonstrated the presence of creatine, phosphocreatine and creatine phosphokinase, in two species of tunicates.

All of these data for the echinoderms as well as the hemichordates are confounded even further by relatively recent findings that creatine phosphate is present in some sponges (Roche and Robin, 1954) and in at least two other invertebrate phyla (Roche *et al.,* 1957). Related comparative biochemical studies by Thoai (1957), by Thoai and Robin (1954), and by Thoai, Roche, Robin and Thiem (1953) supported by a recent report by Hobson and Rees (1955) who identified three new phosphagens of guanidyl derivatives (taurocyamine, glycocyamine and lombricine) in several species of annelids and other invertebrates which the authors suggest serve the same function as creatine in vertebrates.

In passing, one must comment on the use of the hydrolysis reaction alone as the criterion for inferring the presence of either phosphagen, without specific identification of the guanidine as such by some authoritative (hopefully specific) reaction. In this connection, Thoai *et al.* (1953), identified arginine phosphate and creatine phosphate by the usual, classical hydrolysis reactions, but they could not demonstrate the actual presence of released arginine for two species studied by a positive Sakaguchi reaction, nor released creatine, in the one case of *Nereis diversicolor,* by a positive Walpole test.

Finally, the possible implication of the biogenetic principle was tested by studies of the distribution of the two phosphagens in the (unfertilized) ova of the asteroid, *Asterias forbesi,* and the echinoid, *Arbacia punctata,* in relation to their distribution in the adult stages.

At best, the data obtained are equivocal, with creatine and arginine phosphate demonstrable in trace amounts in the ova of *Arbacia punctata,* but in moderate amounts in *Lytechinus* ova. Mende and Chambers (1953) and Chambers and Mende (1953), on the other hand, using the criterion of differential hydrolysis alone, without further separation of the two guanidines, reported the absence of phosphocreatine, but the presence of phosphoarginine by the Sakaguchi test following hydrolysis, in the unfertilized eggs of both *Asterias forbesi* and *Strongylocentrotus dröbachiensis.* The greatest significance of the data obtained in the present study include, first, the presence of creatine in all but two species studied and phosphocreatine in nine species of echinoderms (as well as in the single protochordate species). Secondly, in two species of echinoids, phosphoarginine appears to be replaced by what was formerly considered the phosphagen of the vertebrates, namely phosphocreatine, which occurs in moderately high concentrations in the lantern protractor muscles especially, where its role in energizing of muscle contraction must be logically assumed.

Thus, despite other evidence, some scattered embryological, some biochemical other than that involving the phosphagens (Hyman, 1955, 1959), the data obtained in this present study can only be interpreted as failing to confirm: (1) the hypothesis that invertebrates be considered acreatine, and (2) the existence of a phylogenetic relationship between the primitive chordates and aberrant echinoderms on the basis of biochemical evidence derived from the differential distribution of the two major phosphagens, phosphocreatine and phosphoarginine.

Indeed, the presence of creatine and phosphocreatine in a number of invertebrates, including annelids, as well as the existence of other more or less specific guanidine-derived phosphagens in other phyla like the Annelida, suggests that the distribution of phosphagens, in contemporary species must be only the results of parallel biochemical evolution.

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Summary

1. The arginine, creatine, and related phosphagen content was determined in muscle-containing tissues of nine species of echinoderms, from four subphyla, and in one species of hemichordate. Similar determinations were made on the unfertilized ova of the starfish, *Asterias forbesi,* and of the two urchins, *Arbacia punctata* and *Lytechinus variegatus.*

2. Both phosphoarginine and phosphocreatine occur in moderate to large amounts in the longitudinal muscles of *Thyone briareus* and of *Ludwigthuria floridana.* Both phosphagens occur in light to moderate amounts in the rays of the starfish, *Asterias forbesi, Asterias vulgaris* and *Echinaster sentus,* with light amounts of phosphoarginine and large amounts of creatine phosphate in the rays of the starfish, *Echinaster spinulosa,* and of the brittle star, *Ophioderma brevispina.*

3. Light amounts of phosphoarginine and large amounts of phosphocreatine were also found in the lantern protractors of *Arbacia punctata*.

4. Light amounts of both phosphagens were found in total body homogenates of the hemichordate, *Saccoglossus kowalevskii*.

5. While both phosphoarginine and phosphocreatine are lacking in the unfertilized ova of *A. forbesi*, light amounts of both phosphagens are present in the ova of *L. variegatus* and trace amounts of both in the ova of *A. punctata*.

6. The data obtained fail to confirm (1) the early concept that invertebrates are "acreatinate" and (2) that differential distribution of the phosphagens, as such, can be employed as a criterion for confirming the possible phylogenetic relationship between the echinoderms and primitive chordates.

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